A Simple Algorithm Improves Mass Accuracy to 50-100 ppm for Delayed Extraction Linear MALDI-TOF Mass Spectrometry

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Abstract

A simple mathematical technique for improving mass calibration accuracy of linear delayed extraction matrix assisted laser desorption ionization time-of-flight mass spectrometry (DE MALDI-TOF MS) spectra is presented. The method involves fitting a parabola to a plot of Δm vs. mass data where Δm is the difference between the theoretical mass of calibrants and the mass obtained from a linear relationship between the square root of m/z and ion time of flight. The quadratic equation that describes the parabola is then used to correct the mass of unknowns by subtracting the deviation predicted by the quadratic equation from measured data. By subtracting the value of the parabola at each mass from the calibrated data, the accuracy of mass data points can be improved by factors of 10 or more. This method produces highly similar results whether or not initial ion velocity is accounted for in the calibration equation; consequently, there is no need to depend on that uncertain parameter when using the quadratic correction. This method can be used to correct the internally calibrated masses of protein digest peaks. The effect of nitrocellulose as a matrix additive is also briefly discussed, and it is shown that using nitrocellulose as an additive to αCHCA matrix does not significantly change initial ion velocity but does change the average position of ions relative to the sample electrode at the instant the extraction voltage is applied.

Introduction

Mass calibration accuracy is an important factor for the application of MALDI-TOF MS to biochemical analyses. [1-3] The ability of MALDI-TOF MS to analyze large numbers of peptides simultaneously makes it an ideal tool in the field of peptide mapping. [4-5] The incorporation of delayed extraction (DE), otherwise known as time-lag focusing [3], has improved mass resolution on MALDI-TOF spectra of enzymatically digested proteins significantly [6-8], thus confining mass assignments to a narrower range commensurate with the better resolution. Unfortunately, the use of delayed extraction can result in limited calibration accuracy, especially over large mass ranges.

Calibration of linear (non-reflecting) MALDI-TOF mass spectra is based on the assumption that the relationship between ion time-of-flight (TOF) and the square root of the mass-to-charge ratio (m/z) is linear. [9] When DE is applied, that assumption is no longer valid [3]. Lighter ions arrive at the detector slightly later than a linear calibration would predict, whereas heavier ions arrive at the detector slightly earlier than a linear calibration would predict. Over short mass ranges, this effect is negligible and a linear calibration produces sufficient mass accuracy for most applications. As the mass range of interest widens, however, this effect is magnified causing unacceptable levels of error in mass calibrations. [2] Linear calibrations tend to underestimate the mass of analytes at the extreme ends of the mass range and overestimate the mass of analytes in the middle of the range.

Attempts have been made in the past to correct for these deviations. Juhasz, et al., added second- and third-order terms to his first-order (linear) calibration equation to account for errors that were related to instrument geometry and initial ion velocity. [10] Egelhofer, et al., fit a line to the error data produced from his linear calibrations and used the equation of that line to mathematically adjust his data points closer to theoretical values. [1] Here we report a quadratic correction method to improve the mass accuracy of linearly calibrated DE MALDI-TOF MS data.

Materials and Methods

Alpha-cyano-4-hydroxycinnamic acid (αCHCA), used as a matrix, was purchased from Sigma (St. Louis, MO). Nitrocellulose, used as a matrix additive, was purchased from Schleicher & Schuell (Keene, NH).

The peptides leucine enkephalin, substance P fragment 1-7, bradykinin, substance P, dynorphin A fragment 1-13, melittin, and insulin chain B – oxidized were also purchased from Sigma. The theoretical mass for each of the listed peptides was determined by inputting its amino acid sequence into the Peptide Mass Calculator (http://www.proteometrics.com/javautilities/pepcalc.htm). The mass of one proton was added to the theoretical monoisotopic masses generated from the Peptide Mass Calculator to account for the proton added to the peptides during the ionization event.

Myoglobin from horse heart, cytochrome C from horse, and cytochrome C from cow were purchased from Sigma. Sequencing grade TPCK-modified trypsin was purchased from Promega (Madison, WI). The TPCK modification reduced the amount of trypsin autolysis products in the digest reactions.

Myoglobin and cytochrome C were digested by combining 1 nmol of protein with 0.2 μg of modified trypsin in a 20 μL reaction volume containing 25 mM ammonium carbonate, adjusted to pH 8 using trifluoroacetic acid (TFA). The digestion was allowed to proceed at room temperature for 3-24 hours, after which the reaction was quenched by adding an equal volume of 0.1% TFA. The digest samples were then mixed with matrix as described below with no further cleanup.

Two mass spectrometers were used to analyze the peptide and protein digest samples. The first was a linear MALDI-TOF MS with delayed extraction, built in-house. The details of this instrument are described elsewhere. [11] The sample target for this instrument is a circular stainless-steel probe 4 mm in diameter attached to the end of a long rod that is inserted into the vacuum chamber. The rod is mounted such that the center of the probe is 1.5 mm from the center line of the vacuum chamber, which allows the probe to be rotated to find a fresh spot as needed. For the data collected here, it was rotated after about 50-100 shots. The second instrument was a Voyager Biospectrometry Workstation (PerSeptive Biosystems, Framingham, MA). This instrument was operated in linear mode, with delayed extraction applied. The sample target for this instrument was a 192-spot stainless-steel plate, where the 1-mm diameter spots were surrounded by a hydrophobic surface to contain sample droplets. The samples in both instruments were irradiated in 3-ns bursts with 337-nm light from a nitrogen laser. All experiments were conducted in the positive ion mode.

MALDI samples were prepared by a 2-step process known as the sandwich method. [12] To provide the base layer, a small amount (5 μL for the in-house instrument, 0.3 μL for the Voyager) of matrix solution was deposited on the sample target and allowed to dry. In some cases, the matrix solution was a 20-fold dilution of a saturated solution of αCHCA in acetone. In most cases, the matrix solution contained the same concentration of αCHCA mixed with 2 μg/μL of nitrocellulose in a solution of 4:1 acetone:isopropanol (v/v). For the second layer, peptide and protein digest samples were mixed to concentrations of 0.1-5 μM in a solution containing a 20-fold dilution of a saturated solution of αCHCA in equal volumes of water and acetonitrile containing 0.1% TFA (v/v). An equal volume of this solution was deposited on top of the dried matrix foundation layer and allowed to dry. Samples prepared for the in-house instrument were dried under a stream of nitrogen, whereas samples used in the Voyager were allowed to dry with no assistance from forced air. Samples which used nitrocellulose as part of the matrix foundation layer were washed after the second drying step by depositing a droplet (1-5 μL) of cold 0.1% TFA in water on the sample, allowing it to sit for 30 seconds, and then blowing it off with a stream of nitrogen.

A schematic of a linear TOF MS is shown in Figure 1. For the Voyager instrument, the distance between the sample plate and the extraction grid, otherwise known as the extraction region (d_1), is 2.5 mm. The distance between the extraction grid and the grounded grid entry into the field free flight tube, otherwise known as the acceleration region (d_2), is 17.2 mm. The length of the field free flight tube (L) is 1228.8 mm. For the in-house instrument, these values are 9.5 mm, 27.9 mm, and 1189.6 mm, respectively. For

the Voyager instrument, the acceleration voltage was set to +15 kV, and after the programmed delay time, the extraction grid was reduced to 95% of the sample plate voltage. A guide wire positioned on the beamline axis between the grounded grid and the microchannel plate detector was set to -3 V. Delayed extraction times were between 200-500 ns. Due to the significantly longer extraction and acceleration regions in the in-house instrument, larger voltage drops and longer delayed extraction times were needed. The sample plate was set to +15 kV, and after the appropriate delay, the grid voltage was reduced to 89% of the sample plate voltage. Delayed extraction times on the in-house instrument were between 1800 and 2300 ns. The in-house instrument was not equipped with a guide wire.

Results and Discussion

Effect of Nitrocellulose on initial ion velocity

According to Juhasz, the initial velocity of ions desorbed in a DE MALDI-TOF system can be calculated by observing changes in ion TOF caused by changes in extraction delay times. [10] The equation that describes this is:

$$v_o \approx \frac{\beta}{t}\left(\frac{dt}{d\tau}\right)$$

where v_o is the initial velocity, t is the ion time-of-flight, $dt/d\tau$ is the change in time-of-flight with respect to extraction delay time, and β is a parameter which is determined from instrument geometry and the ratio between the voltages on the sample electrode and extraction grid. In our in-house instrument with a voltage ratio of 89%, $\beta = 0.0356$. For the Voyager with a voltage ratio of 95%, $\beta = 0.0709$.

Knowledge of initial ion velocity is needed in order to calculate masses using the Juhasz calibration equation. The addition of nitrocellulose to matrix solutions could influence initial ion velocity. A comparison of initial velocities of ions desorbed from αCHCA alone and αCHCA with nitrocellulose was conducted for two calibrants, substance P fragment 1-7 (900.5 Da) and dynorphin A fragment 1-13 (1604.0 Da). For the substance P fragment, the initial velocity was calculated to be 300 ± 10 m/s (n=5) when desorbed from αCHCA alone and 310 ± 13 m/s (n=5) when desorbed from αCHCA with nitrocellulose. These initial velocities are not statistically different (Student's T-test). For the dynorphin A fragment, the initial velocity was calculated to be 251 ± 20 m/s (n=4) when desorbed from αCHCA alone and 285 ± 16 m/s (n=4) when desorbed from αCHCA with nitrocellulose. These initial velocities are also not statistically different. Given that previously reported measurements of the initial velocity of MALDI ions desorbed with a 337-nm laser from αCHCA matrix have error values as high as 17% over eighteen measurements [13], it is safe to say that our experiments (both with and without nitrocellulose) yielded results within the currently accepted range of experimental error. The approximation by Juhasz, et al. [10], that ions desorbed from αCHCA have an initial velocity of 300 m/s is valid whether or not the matrix contains nitrocellulose as an additive.

Although estimates of initial ion velocities did not change significantly with the addition of nitrocellulose to the matrix solution, ion flight times were consistently slightly longer for ions desorbed from nitrocellulose-containing matrix than for ions desorbed from αCHCA alone. The substance P fragment exhibited a flight time of 22699.4 ± 0.8 ns (n=5) when desorbed from αCHCA alone and 22701.7 ± 0.3 ns (n=5) when desorbed from αCHCA with nitrocellulose. The dynorphin A fragment exhibited a flight time of 30248.4 ± 0.4 ns (n=4) when desorbed from αCHCA alone and 30249.7 ± 0.8 ns (n=4) when desorbed from αCHCA with nitrocellulose. Statistical analysis (Student's T-test) of these results revealed a 99% probability that the times-of-flight of the substance P fragments were statistically different and an 82% probability that the times-of-flight of the dynorphin A fragments were statistically different. One or two nanoseconds may not seem like a large enough difference in flight time to be significant, especially when collection electronics are digitizing at 2 ns intervals, but such a difference can cause errors of up to 200 ppm in calibration equations and need to be considered with respect to calibration techniques.

The data used to calculate initial velocity and the measurements of times-of-flight were from the same experiments. It appears that assumptions implicit in the Juhasz equation contributed variability to our initial velocity measurements and disguised the slight differences in initial velocity caused by the presence or absence of nitrocellulose. Initial velocity has also been discussed in terms of the density of the MALDI plume and laser intensity, which undoubtedly influences plume dynamics. Our approach will be to rely more heavily on ion flight times and use a calibration that does not rely on initial ion velocity.

Quadratic correction

Five peptides spanning a mass range of 500-3000 Da were measured on our in-house instrument using a nitrocellulose-free matrix, and the TOF results are shown in Table 1. A linear (first-order) calibration of the form:

$$\sqrt{m/z} = At + B$$

where A and B are empirically determined constants and t is the ion time-of-flight, was fit to these data, and the mass values for each of the peptides generated from the calibration are also shown in Table 1. All calibrated mass values deviate from theoretical values by at least 0.9 Da, and the data set shows an average error of 1262 ppm.

Figure 2 shows the error values from the linear calibration (listed in Table 1) plotted against the mass of the peptide calibrants. A systematic deviation from the theoretical values was observed. A parabola, fit to the data in Figure 2, is shown. The quadratic equation that describes the parabola indicates the approximate amount by which the data deviate from expected values. The value of the quadratic equation at each mass abscissa was subtracted from its corresponding linearly calibrated value. The results are shown in Table 2. The accuracy of each mass corrected by the quadratic equation increased dramatically, and all corrected mass values were within 0.12 Da of theoretical values. The average error for the data set was 59 ppm, a 21-fold increase in mass accuracy over linearly calibrated data.

The same time-of-flight data (Table 1) was then calibrated using the equation published by Juhasz [10], which adds second- and third-order terms to the linear calibration. The second- and third-order terms are essentially constants; they do not depend directly on ion time-of-flight, but rather depend on instrument geometry and initial ion velocity. The published value of 300 m/s as the initial velocity of ions desorbed from αCHCA matrix was used in these calculations. The mass values generated using the Juhasz third-order calibration of the data are shown in Table 3. All mass values generated from this calibration are more accurate than corresponding values generated from the linear calibration (Table 1), but they still deviate from theoretical values by at least 0.8 Da and show an average error of 1150 ppm.

Figure 3 shows the error values from the third-order calibration (listed in Table 3) of the five peptides plotted against their respective masses. These data points assume a parabolic shape similar to the error data from the linear calibration (Figure 2). A quadratic equation was fit to these data points as well, and the quadratic equation that describes this parabola was used to mathematically correct the data from Table 3. The results of this quadratic correction are shown in Table 4. Again, a significant improvement in the mass accuracy is observed. All corrected mass values were within 0.12 Da of theoretical values, and the average error for the data set was 60 ppm, a 19-fold increase in mass accuracy over third-order calibrated data.

A comparison of the quadratically corrected data from the linear calibration (Table 2) with the quadratically corrected data from the third-order calibration (Table 4) shows a high degree of similarity. The masses of each peptide calculated by the two different calibration methods differ by as much as 0.23 Da, an average difference of more than 100 ppm. When the quadratic corrections were applied independently to each data set, however, the mass values returned for each peptide were virtually identical. The quadratically corrected mass values from the two separate calibration methods agreed to within 3 ppm with each other, despite the fact that the data points deviated from theoretical values by an average of 60 ppm. This suggests that second- and third-order correction terms may be unnecessary in calibration equations if an

appropriate correction equation is used. Our correction removes the need for knowledge of initial ion velocity, a factor not known to a high level of accuracy [13], from the calibration equation.

Evenly-spaced calibrants

The mass accuracy obtained by use of a quadratic correction can be maximized with careful choice of calibrants. In the examples above, five calibrants were chosen spanning a range of 500-3000 Da. Four of the calibrants, however, were less than 2000 Da, and three of them were within 600 Da of each other. The quadratic equation, being a simple mathematical fit to the parabola which best fits the error data, was weighted to favor the region of the spectrum where there was more data (i.e., the region between 1000-1600 Da). As a consequence, the R^2 value of the quadratic correction was 0.9979.

For subsequent experiments, calibrants were chosen to be spaced more evenly over the mass range of interest. Table 5 shows the time-of-flight and linearly calibrated mass data for four calibrants in the mass range of 900-3500 Da. The calibrants were spaced such that the mass differences between adjacent calibrants were 700-1200 Da, much more evenly spaced than the 300-1800 Da differences observed for the calibrants in the experiment shown in Tables 2-5. The error values for the linear calibration of these four evenly spaced peptides are comparable to the error seen for linear calibrations of the five peptides shown above. However, as shown in Figure 4, the correction parabola fits the error data better than it did for unevenly spaced calibrants and the R^2 value of the resulting quadratic equation rises to 0.9992. Because the correction curve fits the data better than the correction curve shown in Figure 2, the mass data can be corrected to values much closer to theoretical values than those shown in Table 2. The corrected values for the 4-point calibration are shown in Table 6. All corrected masses agree to within 0.05 Da with their theoretical values, and the average error is 19 ppm, a 35-fold increase in mass accuracy over the uncorrected data.

It should be noted that the quadratic correction method requires a minimum of four calibrants to be mathematically reliable. Since a parabola can be fit precisely to any three non-colinear points, use of this method with a three-point calibration would result in a quadratic correction equation with an R^2 value equal to 1, regardless of whether the data points chosen were valid or not. By using at least four calibrants, the R^2 value of the correction equation can be used as a check to make sure that the identification of the calibrant peaks was correct. If the R^2 value deviates significantly from 1, it is likely that a calibrant peak in the spectrum was mis-assigned.

Myoglobin digest samples

Samples of tryptically digested myoglobin were mixed with samples of the 5-peptide calibration mixture described above, which were used as an internal calibration. These samples were analyzed on the Voyager mass spectrometer, with the results illustrated below. Time-of-flight data for the five calibrants is shown in Table 6. This data was used to generate both a first-order (linear) and a third-order (based on the Juhasz equation) calibration curve, and the masses of the calibrants as determined by each calibration are shown in Table 7. The linear calibration resulted in an average error of 236 ppm, whereas the Juhasz calibration resulted in an average error of 150 ppm. Our quadratic correction method was applied to both calibration methods independently, the results of which are shown in Table 8. Again, the corrected mass values from each independent method were almost identical, and the average mass error of the five calibrants was 5 ppm, a 30-fold improvement over the third-order calibration.

A mass spectrum of tryptically digested myoglobin, including the five internal calibrants, is shown in Figure 5. The linear calibration and quadratic correction generated from the five calibrants in the spectrum were applied to the unknown peaks in the spectrum. The corrected masses of the unknown peaks were input into the MS-Fit search engine of Protein Prospector (http://prospector.ucsf.edu/) and a search of the SwissProt database was performed with a tolerance of 100 ppm. Myoglobin from horse was correctly identified as the source of the fragments in this spectrum. Using the fragment masses supplied by Protein

Prospector as the theoretical basis for comparison, the linearly calibrated and quadratically corrected mass values were analyzed for accuracy. The results of this analysis are shown in Table 9. The linearly calibrated myoglobin digest mass data showed an average error of 204 ppm, and had this data been used to search the MS-Fit database, a tolerance of 300 ppm would have been required to identify the protein as horse myoglobin. The quadratically corrected mass data showed an average error of 29 ppm, a 6-fold increase in mass accuracy that permitted a narrower tolerance in the database search process.

Comparison of Cytochrome C species

In the next phase of the experiment, cytochrome C from two different species (horse and cow) were analyzed to determine if this method was adequate to distinguish digests of proteins with highly similar amino acid sequences. The sequences of cytochrome C from the two species are shown in Figure 6. The two sequences differ by only 3 amino acids over the 154-amino acid length of the proteins.

In separate samples, digests of cytochrome C from horse and cow were mixed with samples of the 4 evenly spaced calibrants described above, which were used as internal calibrations. These samples were analyzed on the Voyager mass spectrometer, with the results illustrated below. In each case, a linear calibration was generated, as was a quadratic correction to the linearly calibrated data. The quadratic corrections resulted in masses for the calibrants that were within 10-30 ppm of theoretical values. The resulting list of peaks was input into MS-Fit and the SwissProt database was searched first with a tolerance of 100 ppm, then with 500 ppm. The results for horse and cow cytochrome C are shown in Tables 10 and 11, respectively. The 100-ppm search of the database using mass values from Table 10 resulted in only one hit – the correct identification of horse cytochrome C. The 100-ppm search using mass values from Table 11 resulted in two hits – cow cytochrome C and dog cytochrome C, whose sequences are identical in the regions of the digest fragments. Neither horse cytochrome C nor cow cytochrome C appeared in the search for the opposite species of cytochrome C, meaning that the high degree of specificity afforded by the 100-ppm search tolerances was able to correctly distinguish the two species.

In each list of horse and cow cytochrome C peaks, however, there were two or three peptides whose corrected mass values did not fall within the 100-ppm tolerance. Searches of the SwissProt database with 500-ppm tolerance revealed the theoretical masses for each of these peaks as well. There are several reasons why the peaks were not corrected uniformly to values within 100 ppm error. In the case of the 779.45 Da peaks in each cytochrome C spectrum, the analyte peak fell outside the region spanned by the four calibrants. Mathematically, the correction equation becomes distorted outside the range of points used to generated the equation, so it continues to be important to choose calibrants that encompass the entire range of analyte peaks. In the case of the 964.54 Da peak in the horse cytochrome C spectrum, several shots in the 50-shot averaged spectrum had saturated the detector, resulting in a shift in the peak centroid to a slightly greater time-of-flight. In the case of the 1633.82 Da peaks in each spectrum as well as the 964.54 Da peak in the cow cytochrome C spectrum, accurate times-of-flight were hindered by low peak intensity or interference from neighboring peaks.

Internal vs. External Calibration

We attempted to use the sets of four and five calibrants described above as external calibrants for myoglobin digest samples analyzed by the Voyager mass spectrometer, and the result of a typical attempt is shown in Table 12. Even though the quadratic correction equation generated by the five-point calibration improved the mass accuracy of the calibrants themselves from 207 ppm to 7 ppm, the mass accuracy of myoglobin digest peaks externally calibrated and corrected by this equation were only improved from 297 ppm to 202 ppm. The poorer accuracy obtained with external calibrants was caused by slight shifts in sample-to-sample times-of-flight. Such shifts exist for a variety of reasons, including sample thickness, sample ablation patterns, and sample-plate position. These shifts are sufficient to limit the effectiveness of the quadratic correction method for externally calibrated samples.

Conclusions

We have demonstrated that it is possible to mathematically improve the mass accuracy of data from linear DE MALDI-TOF MS spectra by the use of a quadratic correction equation generated from internal calibrants. This method can improve the mass accuracy over standard calibration methods by factors up to 30, and it eliminates the need to account for the initial ion velocity in calibration equations. The use of evenly spaced calibrants throughout the mass range of interest maximizes the accuracy for fitting a parabola to the error data. This method is particularly useful in the field of peptide mapping, where narrow tolerances of database searches are preferable.

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